Published in final edited form as:

Am J Med Genet B Neuropsychiatr Genet. 2011 July ; 156(5): 517–531. doi:10.1002/ajmg.b.31197.

Replacement of Homologous Mouse DNA Sequence With Pathogenic 6-Base Human *CREB1* Promoter Sequence Creates Murine Model of Major Depressive Disorder

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Abstract

Major Depressive Disorder (MDD) is a leading cause of disability worldwide. Families with Recurrent, Early-Onset MDD (RE-MDD), a severe, familial form of MDD, have provided an important resource for identifying and characterizing genetic variants that confer susceptibility to MDD and related disorders. Previous studies identified a rare, highly penetrant A(-115)G transition within the human *CREB1* promoter that reduced promoter activity *in vitro* and was associated with depressive disorders in RE-MDD families. The development of an etiology-based recombinant animal model for MDD would facilitate the advancement of our limited understanding of the pathophysiology of MDD, as well as the development of improved treatments. Here we report the construction and initial characterization of a congenic mutant C57BL/6NTac mouse model that carries the human pathogenic sequence at the homologous position of the mouse *Creb1* promoter. The recombinant strain exhibited decreases in reproductive capacity and pup survival that may be related to increased infant mortality observed in RE-MDD families; enlargement of the cerebral ventricles; reduced levels of CREB protein in the mouse cerebral cortex, as predicted from transfection experiments employing the pathogenic human *CREB1* promoter; and alterations in two standardized behavioral tests, the forced swim and marble burying tests. These initial findings support the pathogenicity of the human A(-115)G promoter variant, and invite further characterization of this etiology-based recombinant animal model for MDD. Human promoter variants that have highly penetrant effects on disease expression provide an attractive opportunity for creating etiology-based mouse models of human diseases, with minimal disruption of the mouse genome.

Keywords

Depression; Genetics; Mouse; Animal Model

INTRODUCTION

Unipolar depressive disorders are a leading cause of disability worldwide [Lopez et al., 2006]. Twin studies demonstrate that genetic factors typically account for 40% to 70% of the risk for developing MDD, results that are also supported by adoption studies [for review see Zubenko et al., 2001]. Early age of onset of the first major depressive episode and recurrence increase the morbid risk of MDD among family members [Zubenko et al., 2001].

Families identified by individuals with Recurrent, Early-Onset MDD (RE-MDD), a severe and strongly familial form of MDD, have provided an important resource in efforts to identify and characterize genes that contribute to the risk of developing MDD and related conditions that aggregate in these families [Zubenko et al., 2001; Maher et al., 2002]. Interestingly, the rate of infant mortality within these families was several-fold higher than for the local population from which they were recruited, and the rate of mortality from all causes was significantly increased among adult family members [Zubenko et al., 2001].

Model-free linkage analysis of a region of chromosome 2q33-35, highlighted by previous case-control studies [Zubenko et al., 2002c; Philibert et al., 2003] and supported by within-family analyses employing the transmission disequilibrium test [Zubenko et al., 2002b]), has revealed evidence of sex-specific linkage to depressive disorders extending over 15 cM in our 81 RE-MDD families [Zubenko et al., 2002a, 2003b; Maher et al., 2009]. Peak multipoint LOD scores of 6.33 and 6.87 occurred at SSTRPs (simple sequence tandem repeat polymorphism) D2S2321 and D2S2208, respectively. This finding resulted from linkage of the 2q33-35 region to unipolar Mood Disorders among the women in these 81 RE-MDD families that reached genome-wide significance. The 451 kb region between D2S2321 and D2S2208 includes *CREB1*, which encodes a 43 kDa cAMP response element binding protein (CREB) that is a member of the basic leucine zipper family of transcription factors [Mayr and Montminy, 2001].

CREB1 is an excellent candidate for a susceptibility gene that influences the risk of developing MDD and related disorders. *CREB1* is ubiquitously expressed in human tissues and its target genes encode biosynthetic enzymes and receptors for neurotransmitters, neuropeptides, neuronal growth factors, as well as a host of other genes whose products participate in a wide range of cellular functions. Alterations in *CREB1* gene expression and CREB phosphorylation have been reported in clinicopathologic studies of temporal cortex from patients with MDD, in the hippocampus and nucleus accumbens of animal models of MDD and related disorders, and in the brains of rodents exposed to chronic treatment with antidepressant drugs [Nestler et al., 2002; Carlezon et al., 2005]. CREB has also been implicated in neuronal plasticity, cognition, and long term memory [Weeber and Sweatt, 2002], abnormalities of which commonly occur in patients with MDD, may predispose patients to the onset or recurrence of MDD, and may be related to the eventual development of irreversible dementia in some patients [Zubenko, 2000; Zubenko et al., 2001]. Finally, reports of synergistic interactions of CREB with nuclear estrogen receptors [Lazennec et al., 2001; McEwen, 2001; Tremblay and Giguere, 2001] may provide a mechanism by which CREB facilitates sex-specific patterns of gene expression that manifest themselves in the sex-specific effects of risk alleles for unipolar Mood Disorders.

Systematic re-sequencing of the *CREB1* gene in affected members of 81 RE-MDD families has identified rare, highly penetrant sequence variants at positions -656 and -115 that cosegregate with unipolar depressive disorders in two large multigenerational families [Zubenko et al., 2003a] and three small nuclear families [Zubenko and Hughes, 2010], respectively. Transfection experiments that employed constructs containing the wild type (WT) or variant *CREB1* promoters coupled to a reporter gene support the hypothesis that the A₋₆₅₆ allele contributes to the development of MDD in women by selectively increasing the activity of the *CREB1* promoter in brain cell lines exposed to 17 β -estradiol [Zubenko and Hughes 2008, 2009]. Analogous transfection experiments revealed that the G₋₁₁₅ promoter variant reduced promoter activity in CATH.a neuronal cells regardless of the hormonal environment, consistent with the observation that increased risk for unipolar depressive disorders conferred by this allele was *not* limited by sex [Zubenko and Hughes, 2010]. Evidence from an *in silico* analysis suggests that the increased promoter activity conferred by the G to A transition at position -656 may be mediated by the creation of a CP2 binding

site [Zubenko and Hughes, 2008], while the A to G transition at position -115 that decreases promoter activity eliminates a CP2 binding motif [Zubenko and Hughes, 2010]. In summary, the effects of *CREB1* promoter variants on promoter activity, their influence on the development of unipolar Mood Disorders, and the interaction of their phenotypic expression with sex, seem likely to be complex and allele-specific rather than a general property of the *CREB1* locus.

Improving our rudimentary understanding of the underlying pathophysiology of MDD and the mechanism of action of existing antidepressant treatments remains a challenging task, but seems a prerequisite for developing more effective interventions aimed at preventing or reducing the consequences of this major public health problem. The development of an etiologically valid animal model for MDD would significantly accelerate the pace toward achieving these goals. The laboratory mouse has many features that make it an attractive model organism for the study of human disease, including their striking similarity to humans in anatomy, physiology, and genetics. As an example, the mouse and human genes that encode CREB share considerable nucleotide sequence homology, and are both comprised of nine exons including an untranslated first exon (Figure 1A) [NCBI, 2002]. The first 190 bases of the mouse *Creb1* promoter deviate from the corresponding human sequence in only 12 positions, making it possible to unambiguously identify the homologous position corresponding to the human pathogenic A(-115)G variant (Figure 1B). Since the major transcriptional start site for the mouse *Creb1* gene lies 50 bases 3' to that for the human orthologue, the homologous base in the mouse *Creb1* promoter is located at position -165. In this report we describe the construction and initial characterization of a congenic mutant C57BL/6NTac mouse line that carries the homologous human pathogenic sequence at this position.

MATERIALS AND METHODS

Construction of Targeting Vector

The targeting vector (Figure 2A) used for the creation of the mutant mouse line was produced by modifying pNTK [Mortensen, 2008], which was generously provided along with its nucleotide sequence by Dr. Richard M. Mortensen (University of Michigan, Ann Arbor). Two contiguous *Apa*L1 restriction fragments containing the WT C57BL6 *Creb1* promoter and exon 1 (Insert 1, position -4575 to 1716, 6.3 kb), and a portion of the 17.8 kb intron 1 (Insert 2, position 1717 to 6938, 5.2 kb), were identified within BAC clone RP24-528I8 using RestrictionMapper software (www.restrictionmapper.org) and purified by preparative agarose gel electrophoresis. The cohesive ends of mouse Inserts 1 and 2 were modified by the ligation of adapters that contributed unique *Sal*I and *Nhe*I restriction sites, respectively, along with an unpaired 3'A to enable the resulting inserts to be TOPO cloned. Modified Inserts 1 and 2 were gel purified and cloned into plasmids pCR2.1-TOPO and pCR-XL-TOPO (Invitrogen, Carlsbad, CA), respectively. The *Bam*HI site adjacent to the *neo* cassette of pNTK was converted to a *Nhe*I site to provide a unique site into which Insert 2 was subcloned in the opposite transcriptional orientation of *neo* to produce pNTK/*Nhe*I/Insert 2.

Six bases in positions -170 to -165 (5'-CCAGTA-3') in the WT mouse *Creb1* promoter located in Insert 1 were replaced by the homologous six bases from the pathogenic human promoter (positions -120 to -115; 5'-TCCCCG-3') using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The rationale for humanizing the additional 4 bases (one base in this sequence is identical in mouse and human) was based on an *in silico* analysis of the vertebrate transcription factor binding motifs listed in TRANSFAC version 6.0 that identified these bases among motifs whose binding would be altered by the human pathogenic A to G transition at position -115 [Wingender et al., 2001];

Kel et al., 2003]. Briefly, two complimentary oligonucleotides containing the desired bases and flanking plasmid sequence were synthesized (Midland Certified Reagent Co., Midland, TX), annealed to denatured plasmid pCR-XL-TOPO/Insert 1, and extended to produce two complimentary full length single strands. After 15 cycles, the methylated template plasmid was degraded with *DpnI* and the annealed products were transformed into bacteria and grown on selective media. The specified five base changes of the six-base sequence in the mutagenized pCR-XL-TOPO/Insert 1_M were confirmed by automated sequencing (Applied Biosystems, Foster City, CA) performed by the University of Pittsburgh Genomics and Proteomics Core Laboratories.

The mutagenized Insert 1_M was excised from pCR-XL-TOPO/Insert 1_M by digestion with *SalI* and subcloned into the unique *SalI* site adjacent to the *neo* cassette of pNTK/*NheI*/Insert 2 in the same orientation as Insert 2, completing the construction of the targeting vector shown in Figure 2A. The products of each cloning step in the construction process were confirmed by restriction mapping, and the sequence of the targeting vector from positions -1470 to 336, including the *Creb1* promoter and exon 1, was also confirmed by automated DNA sequencing.

Establishment of Recombinant C57BL6/NTac Embryonic Stem (ES) Cell Line

The targeting vector was linearized by cleavage at the unique *NotI* site and electroporated into WT C57BL6/NTac embryonic stem (ES) cells (inGenious Targeting Laboratory, iTL, Stony Brook, NY). Neomycin-resistant clones were isolated and corresponding DNA samples were genotyped to identify clones that carried the altered six-base *Creb1* promoter sequence as the result of the homologous recombination event depicted in Figure 2B.

Genomic DNA from these clones was PCR amplified using a forward primer located within the *neo* gene and a reverse primer located beyond the cloned region of Insert 2 (for primer sequences, see Supplemental Table 1). The production of a 6.5 kb product indicated the presence of *neo* in the predicted location and orientation within intron 1 of the recombinant *Creb1* allele (Figure 2C, left panel). ES clones that produced this result were further evaluated for the presence of the mutagenized bases in the promoter of the recombinant *Creb1* allele. Genomic DNA from these clones was PCR amplified using a forward primer located beyond the boundary of the cloned region of Insert 1 and a reverse primer located within exon 1 (Figure 2C, right panel). The *Creb1* promoter region of the predicted 4.7 kb PCR product was sequenced using an internal forward primer to identify ES clones that were heterozygous for the 5'-TCCCCG-3' sequence at positions -170 to -165 of the recombinant *Creb1* allele (Figure 2D).

Injection of Embryos, Establishment of Chimeras, and Breeding of Congenic Mutant C57BL6/NTac Line

Recombinant C57BL6/NTac ES cells were injected into BALB/c blastocysts and implanted into pseudopregnant females to produce chimeras. Potential founder chimeras were identified by the extent of black coat color and were bred to C56BL6/NTac animals to maintain congenicity with this inbred strain. One male chimera produced black (C57BL6/NTac) offspring that were heterozygous for the altered *Creb1* gene and served as the founder for the congenic mutant line (Figure 3). Heterozygotes were transferred from iTL to The Jackson Laboratory (JAX, Bar Harbor, ME) for re-derivation by *in vitro* fertilization using sperm from male C57BL6/NTac heterozygotes with ova harvested from wild-type C57BL6/NTac females, cryopreservation, speed expansion, and colony maintenance. All animal services were performed at AAALAC-accredited (Association for Assessment and Accreditation of Laboratory Animal Care International) facilities that employ specific-pathogen-free vivaria. Transportation of mice between sites was accomplished by a

specialized service that fulfilled the criteria of both facilities. Mice were provided fresh food and water *ad libitum*. These protocols were approved by the Institutional Animal Care and Use Committees of the State University of New York, Stony Brook (iTL), JAX, and the University of Pittsburgh.

Mouse Genotyping

Genomic DNA was isolated from each 4 mm tail biopsy by incubation in 150 μ l of 100 mM Tris, pH 8.5, 400 mM NaCl, 5 mM EDTA (all Sigma, St. Louis, MO), 0.2% SDS (Gibco, Grand Island, NY), and 0.5 mg/ml Proteinase K (Roche Diagnostics Corp., Indianapolis, IN) for at least 3 hours at 55°C. DNA was precipitated from clarified supernatants by the addition of 270 μ l of 100% ethanol (Pharmco, Brookfield, CT), pelleted by centrifugation, air-dried, and resuspended in 150 μ l of 1 mM Tris, pH 8.0, 0.1 mM EDTA (Sigma). Volumes were reduced by 1/3 for the isolation of DNA from ear biopsies. When required, further purification was achieved by combining 50 μ l of DNA solution with 1 ml of Wizard® DNA Clean-Up Resin followed by packing into a Minicolumn by vacuum (Promega, Madison, WI). The resin was washed with 2 ml of 80% isopropanol (EMD Chemicals, Gibbstown, NJ) and eluted with 50 μ l of nuclease-free water at 80°C (Gibco).

Genotyping for the presence, position, and orientation of the *neo* gene, and automated DNA sequencing of the altered bases located within the *Creb1* promoter (Figure 3) were performed using the same approach as described for ES cells. Genotyping of the *Creb1* promoter was also performed using a PCR-based RFLP (restriction fragment length polymorphism) assay that relied on a *BsmFI* site created by the altered promoter bases (Figure 3C). PCR amplification of a 584 bp region from genomic DNA that included positions -170 to -165 of the *Creb1* promoter was performed using forward and reverse primers described in Supplemental Table 1. Digestion of the PCR product amplified from the WT mouse with *BsmFI* produced 173 bp and 411 bp cleavage products, while digestion of the corresponding PCR amplicon from the homozygous mutant mouse produced 173 bp, 215 bp, and 196 bp fragments. Digestion of the 584 bp amplicon produced from the DNA template of the heterozygote yielded all four cleavage products. Finally, rtPCR and DNA sequence analysis confirmed the absence of *neo* sequence in the *Creb1* transcript expressed in the brains of recombinant mice (see supplementary online material, Supplemental Figure 1).

Protein Electrophoresis and Immunoblotting

Brains were collected from mice at approximately 25 weeks of age. Tissue from the cerebral cortex was homogenized in 1 ml of T-PER® Tissue Protein Extraction Reagent containing 1× Protease Inhibitor Cocktail, 1× Phosphatase Inhibitor Cocktail, 5 mM EDTA (all from Pierce, Rockford, IL), and 1 mM dithiothreitol (Sigma), per 100 mg tissue. After the removal of debris by centrifugation at 10,000 × g for 5 min at 4°C, clarified supernatants were stored at -20°C. Protein concentrations of tissue extracts were determined using the BCA™ Protein Assay Kit (Pierce), using bovine serum albumin (fraction V) as the standard (Pierce). Assays were performed in duplicate and averaged.

Thirty μ l of each tissue extract was resolved on a discontinuous 0.1 % SDS denaturing polyacrylamide gel consisting of a 4 % stacking gel containing 0.375 M Tris, pH 8.8 and a 12 % resolving gel containing 0.125 M Tris, pH 6.8, run in a Protean® II Electrophoresis Cell (Bio-Rad, Hercules, CA). After equilibrating the gel in transfer buffer (48 mM Tris, 39 mM glycine, pH 9.2), the resolved proteins were transferred to an Immobilon-PSQ transfer membrane (Millipore, Billerica, MA) using a Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad).

The remaining protein binding sites on the membrane were blocked by washing in Tris buffered saline (20 mM Tris, 8 % NaCl, pH 7.6) containing 0.1% Tween-20 (TBS/T)(Dako, Carpinteria, CA) and 5% nonfat dry milk for 1 hr. After washing, the membrane was incubated at 4°C over night in TBS/T containing 5 % bovine serum albumin (Sigma) and a 1:1000 dilution of CREB rabbit monoclonal primary antibody (Cell Signaling Technology, Beverly, MA). Unbound primary antibody was removed by washing with TBS/T, and membrane-bound primary antibody was detected by exposure to a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Cell Signaling Technology) for several hours at room temperature. After washing with TBS/T to remove unbound secondary antibody, the membrane was incubated in LumiGLO® Reagent (Cell Signaling Technology), wrapped in plastic wrap, and exposed to Kodak (Rochester, NY) BioMax MR Scientific Imaging Film. Bands were sized by comparison to a Biotinylated Protein Ladder (Cell Signaling Technology) that had been run concurrently with the tissue extracts and detected using a 1:1000 dilution of HRP-conjugated goat anti-biotin antibody (Cell Signaling Technology) that was included during the incubation with the secondary antibody.

Band intensities were quantified using a Pharmacia LKB 2222-020 UltroScan XL Laser Densitometer (Uppsala, Sweden). To permit comparison of the samples across gels, the intensity of each sample band was first normalized by the intensity of a CREB Control Cell Extract (Cell Signaling Technology), a uniform quantity of which was run concurrently on each gel. This control-normalized value was subsequently normalized by the protein concentration of each sample.

3-Dimensional Magnetic Resonance Microscopy (3D-MRM)

MRM was performed using minor modifications of methods described by Koshibu et al. (2005). Following perfusion and fixation, brains were immersed in phosphate-buffered saline in a sealed plastic tube. Each tube was placed into a 15-mm diameter birdcage radio frequency resonator and positioned in an 11.7-T, 89-mm vertical-bore, Bruker AVANCE micro-imaging system. The sample temperature was regulated at 15°C. The image data set was acquired with a diffusion weighted spin-echo sequence (TR/TE = 900/25 ms NA = 4) with the diffusion gradient applied along the rostral-caudal direction and an effective b-value of 1900 s/mm². The data set was acquired with a 256 × 128 × 128 matrix that was zero-filled to 256 × 256 × 256, yielding a final isotropic resolution of 62 μm.

The MRM image data were analyzed using ImageJ software [Rasband, 1997-2011; Abramoff et al., 2004] downloaded from NIH [URL: <http://rsbweb.nih.gov/ij/>] running the Bruker [<http://rsbweb.nih.gov/ij/plugins/bruker.html>] and HandleExtraFileTypes [<http://rsb.info.nih.gov/ij/plugins/file-handler.html>] plug-ins. Image thresholding was used to segment regions of interest (ROIs) and the Analyze Particles tool was used to determine the ROI volumes. Three-dimensional renderings of the brains and ventricles were produced using the 3D Viewer plug-in [Schmid et al., 2010].

Behavioral Phenotyping

Behavioral phenotyping of age and sex-matched young-adult mice that were wild type, heterozygous, or homozygous for the variant *Creb1* promoter was performed at the AAALAC accredited facility at PsychoGenics Inc. (PGI, Tarrytown, NY). Mice were acclimated to the colony room for at least two weeks prior to testing, during which they were examined on a regular basis, handled, and weighed to assure adequate health and suitability for testing and to minimize non-specific stress associated with manipulation. During the course of the study, 12/12 light/dark cycles were maintained with a room temperature between 20 and 23°C and a relative humidity of approximately 50%. Food and

water were available *ad libitum*. Age and sex-matched groups of young adult mice (littermates to the extent possible) were evaluated using a battery of standardized behavioral tests related to “depression and anxiety-like” features by trained staff who were blind to genotype. These included the forced swim test [Porsolt et al., 1977; Sanchez and Meier, 1997], marble burying [Broekamp et al. 1986; Witkin, 2008], the open field test [Bouwknicht and Paylor, 2002; Bouwknicht et al., 2004a,b], stress-induced hyperthermia [Lecci et al., 1990; Olivier et al., 1998; Bouwknicht and Paylor, 2002; Bouwknicht et al., 2004a,b], and tail suspension test [Bernardi et al., 1989; Crowley et al., 2004; Cryan et al., 2004]. The methods for the forced swim test and marble burying test are described below, while the methods and results for the remaining tests are described in the supplementary online materials and at www.psychogenics.com. Approval of the behavioral phenotyping protocols was provided by the Institutional Animal Care and Use Committees at PGI and the University of Pittsburgh.

The forced swim test of each individual mouse consisted of one 6 min session of forced swimming in individual opaque cylinders (15 cm tall × 10 cm wide, 1000 ml beakers) containing fresh tap water at a temperature of 23°C ± 2°C and a depth of 12 cm. The time each animal spent immobile was recorded for each minute of the trial. The mean cumulative immobility time reached a maximum for all three genotypes after approximately 3 min. Immobility was defined as the postural position of floating in the water, generally with the back slightly hunched and the head above water with no movements or with small stabilizing movements of the limbs. After the swim test, each mouse was placed in a pre-heated cage with a heating pad, and allowed to dry for at least 10 min before returning to the home cage.

Marble burying was assessed by placing individual mice in clean cages containing approximately 6 cm of hardwood bedding and twenty black marbles placed in spaced rows of 5 for 30 min. Distance traveled during the test was captured by overhead cameras and quantified using Video Tracker Software (ViewPoint Life Sciences Software, France). After termination of the test, the mice were removed from the cage and the number of buried marbles was counted. A marble was considered buried if it was pushed at least two thirds into the bedding.

Statistical Analyses

Continuous variables are presented as means ± SD or ± SE (for behavior testing results). Mean litter sizes were compared using two-tailed t tests. Categorical data, presented as counts or proportions, were compared using the chi-squared statistic or Fishers exact test, as appropriate. A repeated measures ANOVA was used to evaluate the effects of sex and genotype on weight gain between weeks 2 and 10. Since Mauchley’s test of sphericity for these longitudinal data was significant, the Greenhouse-Geisser adjustment was used to determine the statistical significance of the findings. A two-way ANOVA was used to evaluate the adequacy of age and sex-matching of groups of mice for molecular and behavioral studies. Two-way ANOVAs were used to evaluate the effects of sex and genotype on the relative amounts of CREB protein in cerebral cortex, as well as the performance of mice in standardized behavioral tests. Significant effects detected by two-way ANOVAs were further evaluated by pairwise comparison of means using the LSD post hoc test. Statistical analysis was performed using SPSS Statistics 17 (Chicago, IL).

RESULTS

Breeding, Development, and General Health of Mutant Strain

The founder chimera was produced from the implantation of albino (BALB/c) blastocysts that had been injected with recombinant C57BL/6NTac ES cells as described in the Methods. Heterozygous mutant mice were produced by mating the male founder chimera with C57BL/6NTac females. The mutant strain was re-derived by *in vitro* fertilization (IVF) of WT C57BL/6NTac ova with heterozygous sperm from the mutant strain, followed by implantation of 330 embryos into pseudo-pregnant females. This procedure gave rise to 148 offspring whose sex ratio (80M/68F vs. 1; $\chi^2 = 0.97$, df = 1, $p = 0.32$) and ratio of WT/heterozygotes (81/67 vs. 1; $\chi^2 = 1.32$, df = 1, $p = 0.25$) did not differ from the expected transmission ratios. Of these 148 mice, only 2 manifested developmental abnormalities; one heterozygous female succumbed to hydrocephalus and another was abnormally small and underdeveloped.

Thirty-two breeding units (30 pairs, 2 trios) were established from the remaining IVF-derived heterozygotes at 8-10 weeks of age. Disruptions of the breeding units were minimized to avoid inhibiting mating behavior and proper mothering of newborn pups. Within the subsequent 5 months, by which time the birth rate had markedly declined among the productive breeding units, only 20 (59%) of the 34 females had produced a litter. The observed proportion of breeding units that did not produce offspring, 41%, is over 8 fold greater than the 5% rate reported by Taconic Farms [personal communication] for WT C57BL/6NTac breeding pairs of similar age ($\chi^2 = 11.769$, df = 1, $p = 0.0006$). Twenty heterozygous females produced a total of 60 litters consisting of 321 pups, 29 (9%) of which died within the first few weeks. The mean numbers of pups delivered/litter and pups surviving were $5.4 \pm \text{SD } 2.2$ and $4.9 \pm \text{SD } 2.4$, respectively. These values were below the corresponding 95% confidence intervals, 7.4 - 8.0 and 5.5 - 6.3, reported for WT C57BL/6NTac mice [Whitaker et al., 2007]. Although most of the losses of newborn mice were accompanied by cannibalization by the mother, sufficient tissue was recovered from 13 dead pups to make genotyping possible. These 13 included 1 WT, 11 heterozygotes, and 1 homozygote, a sample too small for drawing reliable inferences about whether pup genotype affected survival (expected ratio of 1/2/1, Fisher's exact $p = 0.31$). The remaining 56 litters included 292 pups whose observed and expected distributions by sex and genotype are shown in Table 1. The observed M/F ratio of 0.62 was significantly below the expected ratio of 1 ($\chi^2 = 15.84$, df = 1, $p = 6.9 \times 10^{-5}$). The observed ratio of 1.0/1.7/0.3 for WT/heterozygotes/homozygotes also differed significantly from the expected Mendelian ratio of 1/2/1 ($\chi^2 = 33.53$, df = 2, $p = 5.2 \times 10^{-8}$), reflecting the reduced transmission of the *Creb1* promoter variant and a 3-fold reduction in the proportion of homozygotes among the F2 progeny.

Six breeding pairs consisting of male and female homozygotes of similar age (mean age $9.1 \pm \text{SD } 1.0$ wks) were established to further evaluate the observed effect of genotype on reproduction and pup survival. Copulation occurred in all six cases within 6 days, as determined by vaginal plugging. Pregnancy was assessed at days 11 and 16 after plugging. In all but one case, females judged to be pregnant by palpation subsequently delivered a litter. Three (50%) of the 6 breeding pairs produced 6 litters totaling 16 pups in the ensuing 2 months, although only 2 pups survived to weaning. The mean numbers of pups delivered/litter and pups surviving to weaning/litter were $2.7 \pm \text{SD } 1.2$ and $0.3 \pm \text{SD } 0.8$, respectively. These values were significantly lower than the corresponding means, $5.4 \pm \text{SD } 2.2$ ($t = 2.98$, df = 64, $p = 4.1 \times 10^{-3}$) and $4.9 \pm \text{SD } 2.4$ ($t = 4.48$, df = 64, $p = 3.2 \times 10^{-5}$), for the F2 (het $\times$ het) cross described in the previous paragraph, and substantially below the corresponding 95% confidence intervals, 7.4 - 8.0 and 5.5 - 6.3, reported for WT C57BL/6NTac mice [Whitaker et al., 2007].

No differences in appearance or behavior were observed among the surviving heterozygous or homozygous mutant mice during handling or routine colony maintenance (Figure 4, top panels), and examinations of the gross anatomy of internal organs, including the brain, during necropsies revealed no focal abnormalities. As shown in Figure 4 (bottom panels), age ($F = 4223.31$; $df = 3.42, 553.68$; $p = \text{too small to calculate}$), age $\times$ genotype ($F = 2.97$; $df = 6.84, 553.68$; $p = 5.0 \times 10^{-3}$), and age $\times$ sex ($F = 86.84$; $df = 3.42, 553.68$; $p = 7.1 \times 10^{-51}$), all had significant effects on weight during weeks 2 through 10 (all results from repeated measures ANOVA with adjustments of df using Greenhouse-Geisser method). Modest ($\sim 10\%$) reductions in the body weights of both male and female homozygotes were detected during this transition from newborn to young adulthood (homozygote vs. WT, $p = 1.3 \times 10^{-6}$; homozygote vs. heterozygote, $p = 8.0 \times 10^{-8}$; LSD post hoc comparisons).

Three-Dimensional Magnetic Resonance Microscopy (3D-MRM) of Mouse Brains

Three-dimensional translucent brain images highlighting the exterior surface and ventricles of male and female adult WT and homozygous recombinant C57BL/6NTac mice are presented in Figure 5. The images of the WT brains closely resemble those depicted in the mouse brain atlas of Paxinos and Franklin [2001]. Corresponding movies that rotate these images around the vertical axis provide further anatomic detail and have been included in the Supplementary Online Materials. Neither sex nor genotype was associated with notable alterations in the external appearance or volumes of the intact brains (F WT, 362.5 mm^3 ; F Hom, 336.9 mm^3 ; M WT, 339.9 mm^3 ; M Hom, 332.4 mm^3). In contrast, the cerebral ventricles of brains from the homozygotes (F, 8.04 mm^3 ; M, 10.12 mm^3) were 2.7 times larger in volume than those from WT mice of the same sex (F, 2.99 mm^3 ; M, 3.79 mm^3). This genotype effect appeared to be more pronounced for the lateral and third ventricles than for the fourth ventricle.

Effect of *Creb1* Promoter Genotype on CREB Protein Levels in Cerebral Cortex

Since transfection experiments revealed that the human pathogenic G₋₁₁₅ promoter variant reduced *CREB1* promoter activity in both neuronal and glial cell lines [Zubenko and Hughes, 2010], we hypothesized that the homologous mutation would reduce the level of CREB protein in the mouse cerebral cortex. To test this hypothesis, cortical tissue was collected from six age-matched adult mice of each sex and genotype (mean ages 25.3 to 25.5 weeks), and the amounts of CREB protein in clarified homogenates was determined by electrophoresis and immunoblotting, as described in the Methods. Immunoblots revealed a single protein species with a molecular weight of $\sim 43 \text{ kDa}$, consistent with that reported for CREB [Mayr and Montminy, 2001].

As shown in Figure 6, genotype had a significant effect on the amount of CREB protein (2-Way ANOVA: $F = 20.96$; $df = 2, 30$; $p = 2.0 \times 10^{-6}$). This effect reflected 44% and 40% reductions in the mean amount of CREB in the cerebral cortices of homozygotes compared to the corresponding values for WT ($p = 1.5 \times 10^{-6}$, LSD post hoc test) and heterozygous mice ($p = 1.5 \times 10^{-5}$, LSD post hoc test), respectively. The mean amounts of CREB protein in the cerebral cortices of WT and heterozygous mice were similar ($p = 0.43$, LSD post hoc test). Sex had no effect on the amount of CREB protein in the cerebral cortex (2-Way ANOVA: $F = 2.17$; $df = 1, 30$; $p = 0.15$), nor was a genotype $\times$ sex interaction detected (2-way ANOVA: $F = 0.21$; $df = 2, 30$; $p = 0.81$). This last finding is consistent with the lack of a sex-specific effect of the pathogenic *CREB1* allele on risk for unipolar depressive disorders and the absence of an effect of gonadal steroids on the activity of the results of the transfection experiments that employed the same human *CREB1* promoter variant in noradrenergic neuronal cells *in vitro* [Zubenko and Hughes, 2010].

Effect of *Creb1* Promoter Genotype on “Depression-Related” Behavioral Phenotypes

The human pathogenic G₋₁₁₅ promoter variant was identified in studies of families identified by probands with RE-MDD, for which early-onset of MDD was defined as occurring at or before age 25 with at least one depressive episode after age 18. The mean age at onset of the first major depressive episode among the probands from these families was $17.5 \pm \text{SD } 4.4$ years [Zubenko et al., 2001]. Behavioral phenotyping of mice was performed using young adult mice of about 10 weeks of age who were in the corresponding stage of their life cycle. Age and sex-matched groups of WT, heterozygous, and homozygous mice (littermates to the extent possible) were evaluated using a battery of standardized behavioral tests related to “depression related” features by trained staff who were blind to genotype (Figure 7), as described in the Materials and Methods and Supplemental online material.

The forced swim test is based on the psychological hypotheses of behavioral despair and learned helplessness [Porsolt et al., 1977; Sanchez and Meier, 1997]. When rodents are forced to swim in a confined vessel, they ultimately cease attempting to escape, thereby assuming a state of immobility that is remediable by the administration of medications that have antidepressant properties in humans. The mean cumulative immobility time reached a maximum for all three genotypes after approximately 3 min. As shown in Figure 7 (top), genotype had a significant effect on cumulative immobility (2-way ANOVA: $F = 5.30$; $df = 2, 72$; $p = 7.1 \times 10^{-3}$), reflecting greater cumulative immobility of the homozygotes compared to WT ($p = 2.6 \times 10^{-3}$, LSD post hoc test) and heterozygous mice ($p = 1.5 \times 10^{-2}$, LSD post hoc test). Sex did not affect cumulative immobility in the forced swim test (2-way ANOVA: $F = 4.1 \times 10^{-2}$; $df = 1, 72$; $p = 0.84$), nor was a genotype $\times$ sex interaction detected (2-way ANOVA: $F = 1.40$; $df = 2, 72$; $p = 0.26$).

Marble burying by mice has been reported to model anxiety and obsessional/compulsive features, and is reduced by medications that have anxiolytic or antidepressant properties in humans [Broekamp et al., 1986; Witkin, 2008]. The number of buried marbles was counted after termination of the test. As shown in Figure 7 (middle), genotype had a significant effect on marble burying (2-way ANOVA: $F = 3.85$; $df = 2, 72$; $p = 2.6 \times 10^{-2}$), reflecting a reduced mean number of marbles buried by homozygotes compared to WT ($p = 3.8 \times 10^{-2}$, LSD post hoc test) and heterozygous mice ($p = 1.3 \times 10^{-2}$, LSD post hoc test). Sex did not have a significant effect on marble burying (2-way ANOVA: $F = 3.63$; $df = 1, 72$; $p = 6.1 \times 10^{-2}$), and no genotype $\times$ sex interaction was detected (2-way ANOVA: $F = 0.72$; $df = 2, 72$; $p = 0.49$). Neither genotype (2-way ANOVA: $F = 1.33$; $df = 2, 68$; $p = 0.27$) nor sex (2-way ANOVA: $F = 2.03$; $df = 1, 68$; $p = 0.16$) had a significant effect on the distance traveled during the 30 min test (Figure 7, bottom). This last finding indicates that the effects of genotype on mouse performance in the forced swim and marble burying tests were not simply attributable to reduced locomotor activity.

No significant effects of genotype or genotype $\times$ sex interactions were observed in the open field test, stress-induced hyperthermia, or tail suspension test, after correction for multiple comparisons (methods and data are presented as supplementary online material). Furthermore no differences in appearance, locomotion, or other behaviors were identified among the heterozygous or homozygous mutant mice during handling or routine colony maintenance.

DISCUSSION

Choice of Inbred Strain and Congenicity

The C57BL/6 mouse inbred strain has been widely used for studies of mammalian genetics, neurobiology, and behavior. These mice display average performance among inbred mouse strains in most behavioral domains including standardized behavioral tests used for

characterizing “depression-like” features in mice that are remediable by the acute administration of medications that have antidepressant properties in humans with MDD [Porsolt et al., 1977; Broekamp et al. 1986; Bernardi et al., 1989; Sanchez and Meier, 1997; Olivier et al., 1998; Crawley, 2000; Bouwknecht and Paylor, 2002; Bouwknecht et al., 2004a,b; Crowley et al., 2004; Cryan et al., 2004; Witkin, 2008]. As a result, they provide the opportunity to detect changes in test performance without encountering constraints at either extreme of the response range (ceiling or basement effects). In addition, the unique availability of the genomic sequence of this inbred mouse strain at NCBI [2002], as well as the availability of the corresponding BAC clone containing the mouse *Creb1* region, were essential for constructing the recombinant mouse model. Substrains C57BL/6NTac and C57BL/6J are similar in their performance in behavioral tests and their genotypes have been reported to differ at only 0.8% of SNP (single nucleotide polymorphism) loci [Mekada et al., 2009]. However, unlike the C57BL/6NTac substrain, the BL/6J substrain carries a functional deletion of the nicotinamide nucleotide transhydrogenase (*Nnt*) gene that results in abnormalities of glucose and mitochondrial metabolism that could have confounded our mouse model [Mekada et al., 2009].

From the genetic engineering of C57BL/6NTac ES cells to the breeding of chimeras and establishment of the mutant line, congenicity with the BL/6NTac substrain was maintained to minimize unwanted effects of inter-strain genetic variation on the phenotypes of interest [Banbury, 1997]. This consideration is especially important for the genetic background of the ES cells because inter-strain genetic variations tightly linked to *Creb1* would not be separable from the promoter variant by recombination during subsequent breeding. This would have made it impossible to distinguish phenotypic differences attributable to the 5'-TCCCCG-3' sequence at positions -170 to -165 of the recombinant *Creb1* allele from those that potentially resulted from tightly-linked inter-strain genetic variation, or their interaction.

To preserve the congenicity of the mutant strain, the *neo* cassette was not removed using Cre/LoxP or Flp/rt recombination methods [Simon et al., 2009] because C57BL/6NTac strains expressing the respective recombinase genes were not available. Instead, the potential influence of the *neo* cassette on *Creb1* expression was minimized by its localization 1.6 kb into the 17.8 kb intron 1 and orientation in the reverse transcriptional direction. Consistent with this goal, no *neo* sequence was detected in the *Creb1* transcript and the CREB protein detected in the cerebral cortex was of the expected 43 kDa size.

Support for the Validity of the Recombinant Mouse Model of MDD

C57BL/6 mice that are homozygous for null *Creb1* mutations die of respiratory failure in the neonatal period [Rudolph et al., 1998]. In contrast, the conversion of a 6-base sequence in the mouse *Creb1* promoter to the homologous pathogenic sequence identified by genetic studies of RE-MDD produced viable mice expressing phenotypes reminiscent of this human disorder.

Mice of either sex that were homozygous for the mutant *Creb1* allele manifested enlargement of the cerebral ventricles (especially the lateral and third ventricles), suggesting an abnormality of brain development, without obvious alterations in external brain appearance or total brain volume. The evidence for ventricular enlargement in patients with mood disorders is mixed [for review see Savitz and Drevets, 2009], although imaging studies of subjects with RE-MDD are lacking. Decreases in the survival of pups produced by breeding pairs that were heterozygous or homozygous for the mutant *Creb1* allele may be related to the several-fold increase in infant mortality observed in RE-MDD families [Zubenko et al., 2001]. Homozygous mice exhibited no alteration of the sequence of the *Creb1* transcript at the exon1- exon2 boundary, or the size of the CREB protein produced in the cerebral cortex. However, homozygous mice did manifest reduced levels of CREB

protein in the cerebral cortex, with no effect of sex, as predicted by published transfection experiments employing human *CREB1* promoter-reporter gene constructs [Zubenko and Hughes, 2010]. Homozygous mice demonstrated increased immobility in the forced swim test and reduced marble burying. These behavioral studies revealed no evidence of a genotype-sex interaction, consistent with the lack of sex-specific effects of the homologous human promoter variant on unipolar depressive disorders in RE-MDD families, as well as the published transfection experiments [Zubenko and Hughes, 2010]. While the relevance of these behavioral phenotypes to “depression or anxiety-related” features of human psychiatric disorders is controversial, our results revealed statistically significant effects of genotype on these established, standardized behavioral tests that do not appear to be attributable to an impairment or reduction in locomotion. These findings support the pathogenicity of the human promoter variant as a risk factor for unipolar depressive disorders, and affirm the congenic mutant strain as a model of MDD worthy of further investigation. Future studies will extend the characterization of this recombinant mouse model by evaluating the performance of larger numbers of animals in a broader array of behavioral tests including an assessment of learning and memory, pharmacologic remediation of identified behavioral abnormalities, and anatomic, functional, and molecular studies of the brain.

Experimental approaches that employ etiology-based animal models would provide an important addition to studies of MDD that are constrained by limitations inherent in investigations of human subjects and the human central nervous system in particular. Support for the etiologic (construct) validity of our mouse model of MDD results from its reliance on the substitution of a rare, highly penetrant, pathogenic human *CREB1* promoter sequence for the homologous mouse sequence, rather than paradigms based on psychological hypotheses or antidepressant-responsiveness. The use of animal models whose development is based on pre-existing etiologic hypotheses to elucidate the unknown pathophysiology of a disorder can be expected to provide evidence to support the hypotheses embodied in the model. Furthermore, the most effective treatments for human disorders usually address root causes rather than symptomatic manifestations. Over-reliance on preclinical models that have been validated chiefly by responsiveness to existing antidepressant drugs has the potential to provide misleading information on the pathophysiology of MDD. For example, studies of the pharmacologic mechanism of action of diuretics, common treatments for the manifestations of congestive heart failure, would not only provide little insight into the pathophysiology of congestive heart failure, they would implicate the wrong organ. Approaches to experimental therapeutics of MDD that employ etiology-based models also provide an alternative to the circularity of current preclinical models that rely on responsiveness to existing antidepressant drugs, a strategy that seems more likely to identify “me-too” drugs rather than compounds with novel mechanisms of action that extend the effectiveness of available treatment options for MDD and related conditions.

Role of Rare Genetic Variants with Large Clinical Effects in Creating Etiologically Valid Recombinant Models of Disease

The genetic architecture of MDD and other human disorders whose genetic transmission is complex appears to involve contributions from relatively common alleles that have modest effects on the risk of expression, as well as rare alleles that confer a high likelihood of expression. In our studies of families identified through probands with RE-MDD, a severe and strongly familial form of MDD, single base transitions at positions -115 and -656 of the human *CREB1* promoter reflect examples of the latter category [Zubenko et al., 2003a; Zubenko and Hughes, 2008, 2009, 2010]. While these genetic variants are rare, a substantial body of evidence suggests that alteration of CREB signaling may be relevant to the

pathophysiology, clinical presentation, and treatment of a larger fraction of MDD cases, as described in the Introduction. It has been estimated that about 1% of single base-pair substitutions causing human genetic diseases occur within promoter regions, where they usually decrease or increase gene expression by reducing the binding affinity of existing transcription factor binding motifs or creating new ones [Cooper, 2002; de Vooght et al., 2009]. In addition to MDD, pathogenic promoter variants have been reported to cause other CNS disorders including early-onset Alzheimer's disease [Theuns et al., 2006] and familial Parkinson's disease [Le et al., 2003], and to affect the development, course, and treatment responsiveness of a number of systemic disorders.

The promoter sequences of orthologous human and mouse genes often manifest high homology for about 150-200 bases upstream of the initiation of transcription, providing targets for homologous recombination. In these regions, the bases in a mouse promoter that correspond to human pathogenic promoter variants can often be unambiguously identified. Therefore, human promoter variants that have highly penetrant effects on disease expression offer an attractive opportunity for creating etiology-based models of these human diseases with minimal disruption of the mouse genome, analogous to the mouse model for MDD described here.

A large portion of the published literature on mouse models of human disease relies on either a) transgenic models that incorporate transgenes that express proteins (often foreign or mutated) at high levels and under nonphysiological control, at one or more (often undetermined) nonhomologous insertion sites, events that are themselves mutagenic; or b) deletions ("knockouts") of genes whose products are hypothesized to play a role in the etiology of the disease of interest. In the latter case, the resulting models are biased toward providing evidence supporting the etiologic hypothesis employed in the creation of the model. Furthermore, knock-out models often produce truncated proteins, resulting from the deletion of one or more internal exons, and may have residual, unpredictable effects (usually unexplored) that limit the usefulness of the model. Despite the importance of controlling for the effects of genetic background, few if any of published recombinant mouse models of human disease are congenic, especially in chromosomal regions adjacent to the recombination event of interest. The method of creating etiology-based mouse models for human diseases illustrated in our manuscript reduces or eliminates these limitations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by research project grant MH47346 from the National Institute of Mental Health (GSZ), a grant from the Provost's Fund for Research Development, University of Pittsburgh (GSZ), and the Shane Richard Brown Fund, University of Pittsburgh. MRI data were collected at the Pittsburgh NMR Center for Biomedical Research (P41EB001977) at Carnegie-Mellon University. MRI data were analyzed with support from the Office of the Senior Vice Chancellor for the Health Sciences, University of Pittsburgh, and Grant Number UL1 RR024153 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH) and NIH Roadmap for Medical Research. The contents do not necessarily represent the official view of NCRR or NIH.

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Figure 1.

Diagram of the mouse *Creb1* gene (A), and homologous mouse and human promoter sequences (B). **A.** Like the human orthologue, the mouse gene contains nine exons, including an untranslated exon1, and 8 introns. The first intron is 17.8 kb in size. Bases are numbered with respect to the transcription start site. The position in the mouse promoter (-165) that corresponds to the human pathogenic A(-115)G variant is shown. Data were obtained from NCBI sequence viewer *Mus musculus* strain C57BL/6J, Chr 1, (NCBI Build 35.1). The diagram is not drawn to scale. **B.** Identical bases in the mouse (above) and human (below) sequences are indicated by dots in the human sequence to illustrate homology. Bases are numbered with respect to the major transcription start sites that begin at highlighted As (+ strands, 5' to 3'). The position in the mouse promoter (-165) that corresponds to the human A(-115)G variant is shown. Mouse and human DNA sequences were obtained from RefSeq contigs NT_039170 and NT_005403, respectively (NCBI Build 35.1). (In Build 37.2, the transcriptional start site of the mouse *Creb1* gene was relocated 94 bases in the 5' direction, with no change in the DNA sequence. Accordingly, the mouse base that corresponds to the human -115 position would become position -71. This change has no impact on the results presented in this report.)

Figure 2.

Illustration of the targeting vector (A), homologous recombination event (B), and recombinant C57BL/6NTac ES cells (C, D). **A.** The targeting vector containing mouse (purple) and pNTK (blue) sequences was constructed as described in the Methods. Transcribed regions are shown with thick arrows indicating the direction of transcription. Key features include the six-base human *CREB1* promoter sequence containing the pathogenic G variant at mouse position -165, mouse *Creb1* exon 1, *neo* cassette, and restriction sites used for subcloning the mouse inserts and linearizing the vector. **B.** This diagram illustrates the homologous recombination event between the mouse wild-type *Creb1* gene and the targeting vector to produce the desired recombinant C57BL/6NTac embryonic stem (ES) cells. The positions of the forward and reverse PCR primers used to

identify this recombinant are shown as small arrows above the corresponding sequences (for primer identification and sequences see Supplemental Table 1). **C.** Electropherograms showing the results of a PCR-based assay for the homologous recombination event depicted in panel B. PCR amplification of genomic DNA from recombinant C57BL/6NTac embryonic stem (ES) cells was performed using a forward primer located within *neo* (primer 4) and a reverse primer located beyond the 3' end of Insert 2 (primer 6) (left panel). The predicted 6.5 kb PCR product is shown in the right lane. Substitution of targeting vector (TV) DNA, which lacked homology to the reverse primer, or wild-type (WT) mouse genomic DNA, which lacked homology to *neo*, as PCR templates provided negative controls (middle lanes). A molecular weight standard (MW, 1 kb ladder) was included in the left lane. PCR amplification was also performed using a forward primer (primer 1) located beyond the 5' end of Insert 1 and a reverse primer located within exon 1 (primer 3) (right panel). The predicted 4.7 kb PCR product amplified from recombinant ES genomic DNA is shown in the right lane. Substitution of wild type (WT) mouse genomic DNA, which contained identical areas of homology to both primers, also resulted in a 4.7 kb product (lane 3). Substituting targeting vector (TV) DNA, which lacked homology to the forward primer, as the PCR template provided a negative control (lane 2). A molecular weight standard (MW, 1 kb ladder) was included in the left lane. **D.** Automated DNA sequencing traces of positions -176 to -159 span the wild-type *Crebl* promoter (left) and the heterozygous *Crebl* promoter alteration in the recombinant ES cells (right). DNA sequencing was performed using forward primer 2 and the 4.7 kb PCR product amplified from either WT mouse (left) or recombinant ES genomic DNA (right), as shown in the right electropherogram of panel C.

Figure 3.

Photographs of the founder chimera (A) and first C57BL/6NTac heterozygous offspring (B), and mouse genotyping results (C-G). **C.** Electropherogram illustrating RFLP genotyping of the A(-165)G variant. A PCR product spanning position -165 was amplified from genomic DNA using primers 2 and 3 (Supplemental Table 1). The resulting PCR product was digested with *BsmFI* and the digestion products were sized by electrophoresis. The WT strain (E) produced 173 bp and 411 bp fragments; the heterozygote (F) produced 173 bp, 196 bp, 215 bp, and 411 bp fragments; and the homozygous mutant (G) produced 173 bp, 196 bp, and 215 bp fragments. A 100 bp ladder was included as a molecular weight standard (M). **D.** Electropherogram illustrating a PCR-based assay for the presence of *neo* within intron 1. PCR amplification of genomic DNA from mice that were WT (E), heterozygous

(F), or homozygous (G) for the *Creb1* promoter variant at position -165 was performed using a forward primer located within *neo* (primer 5) and a reverse primer located beyond the 3' end of Insert 2 (primer 6). The predicted 5.7 kb PCR product was produced from mice that carried the mutant *Creb1* promoter allele (lanes F, G). Wild-type mouse genomic DNA (E), which lacked homology to *neo*, provided a negative result. A 1 kb ladder was included in the outside lanes as a molecular weight standard (M). E, F, and G. Automated DNA sequencing traces of positions -175 to -160 confirm the genotypes (sequence and position) of the WT (E), heterozygous (F), and homozygous (G) mice. DNA sequencing was performed using forward primer 2 and the 4.7 kb PCR product amplified from *Creb1* genomic DNA, as shown in the right electropherogram of Figure 2, panel C. The six-base human pathogenic sequence and homologous WT mouse sequence are highlighted in traces from the WT and homozygous mutant mice (E, G).

Figure 4.

Trios (wt, het, hom) of male (top left) and female (top right) F2 littermates with weight data for weeks 2-10 (bottom left and right). As shown in the photographs, the *Creb1* promoter genotypes of young adult mice were not identifiable by gross appearance. Weekly mean weights are presented $\pm$ SD (vertical bars), beginning at 2 weeks of age. Symbols for genotype groups at each week were slightly offset to make SD values legible. Age, age $\times$ genotype interaction, and age $\times$ sex interaction all had significant effects on weight during weeks 2 through 10 (repeated measures ANOVA, see text). Modest ($\sim 10\%$) reductions in the body weights of both male and female homozygotes were detected during this interval (homozygote vs. WT, $p = 1.3 \times 10^{-6}$; homozygote vs. heterozygote, $p = 8.0 \times 10^{-8}$).

Figure 5. Three-dimensional magnetic resonance microscopy (3D-MRM) images of brains from male (top) and female (bottom) adult wild type (left) and homozygous recombinant (right) C57BL/6NTac mice. Translucent brain images illustrate the brain surface with underlying ventricles highlighted in white. All four images are shown at the same magnification (2mm scale bar).

Figure 6.

Effect of *Creb1* promoter genotype on CREB levels in cerebral cortex of F2 adult mice. Cortical tissue was collected from six age-matched adult mice of each sex and genotype, and the amounts of CREB protein in clarified homogenates were determined by electrophoresis and immunoblotting. The resulting immunoblots (representative example, above) identified a 43 kDa species consistent with the reported size of the CREB protein, as determined by comparison to a biotinylated protein ladder (not shown). No other immunoreactive species was detected. Bands were quantified by densitometry and the normalized means ($\pm$ SD) are presented in the histogram (below). Significant 44% and 40% reductions in the mean amount of CREB in the cerebral cortices of homozygotes were observed compared to the corresponding means for WT ($p = 1.5 \times 10^{-6}$) and heterozygous mice ($p = 1.5 \times 10^{-5}$), respectively. The mean amounts of CREB protein in the cerebral cortices of WT and heterozygous mice were similar. Sex did not affect the amount of CREB protein in the cerebral cortex, nor was a sex $\times$ genotype effect detected. Significant pairwise differences in means are indicated by asterisks.

Figure 7.

Effects of *Creb1* promoter genotype on performance in the forced swim and marble burying tests. Age and sex-matched groups of WT (16M/16F, mean age = 10.2 wks $\pm$ SD 1.6 wks), heterozygous (Het, 16M/16F, 10.2 wks $\pm$ SD 1.6 wks), and homozygous (Hom, 8M/6F, 10.3 wks $\pm$ SD 1.7 wks) young adult mice (littermates to the extent possible) were evaluated using semi-automated methods by trained staff who were blind to genotype. As shown in the top histogram, homozygotes displayed greater immobility in the forced swim test compared to WT ($p = 2.6 \times 10^{-3}$) or heterozygous mice ($p = 1.5 \times 10^{-2}$). As shown in the middle histogram, homozygotes buried fewer marbles than WT ($p = 3.8 \times 10^{-2}$) or heterozygous mice ($p = 1.3 \times 10^{-2}$). Sex did not have a significant effect on either immobility in the forced

swim test or marble burying, and no genotype $\times$ sex interaction was detected in either test. Neither genotype nor sex had a significant effect on the distance traveled during the marble-burying test (bottom). Mean values for the behavioral tests are presented $\pm$ SEM. Significant pairwise differences in means are indicated by asterisks.

Table 1

Distribution of F2 Progeny by Sex and Genotype.

	Observed Values			Expected Values		
	Male	Female	Total	Male	Female	Total
Wild Type	34	61	95	36.5	36.5	73
Heterozygote	64	102	166	73	73	146
Homozygote	14	17	31	36.5	36.5	73
Total	112	180	292	146	146	292

The observed M/F ratio of 0.62 was significantly below the expected ratio of 1 ($\chi^2 = 15.84$, $df = 1$, $p = 6.9 \times 10^{-5}$). The observed ratio of 1.0/1.7/0.3 for WT/heterozygotes/homozygotes also differed significantly from the expected Mendelian ratio of 1/2/1 ($\chi^2 = 33.53$, $df = 2$, $p = 5.2 \times 10^{-8}$), reflecting the reduced transmission of the *Creb1* promoter variant and a 3-fold reduction in the proportion of homozygotes among the F2 progeny.